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Activated CD56$^+$ lymphocytes (NK+NKT) mediate immunomodulatory and anti-viral effects during Japanese encephalitis virus infection of dendritic cells *in-vitro*

Harini Sooryanarain, Vijay Ayachit, Milind Gore *

Encephalitis Group, National Institute of Virology, Sus Road Campus, Pashan, Pune-411 021, India

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ABSTRACT

Japanese encephalitis virus (JEV) remains one of the major causative agents of pediatric encephalitis. Interaction of dendritic cells (DCs) with innate lymphocytes (NK and NKT) represents a crucial event during anti-viral innate immune response. In the current study, we have tried to understand the interaction between JEV, human monocyte derived DCs (MDDCs), and CD56$^+$ cells (NK+NKT) *in-vitro*. We have used two JEV strains (i) JE057434 (neurovirulent, wild-type) and (ii) SA14-14-2 (non-neurovirulent, live-attenuated vaccine) to investigate the effect of viral virulence on the functional status of primary human MDDCs. Our preliminary results indicate that replicating JEV induces MDDCs maturation via PI3K and p38 pathways. We also show that the presence of IL2-activated CD56$^+$ cells impart both immunomodulatory and anti-viral effects on DCs infected with JEV. Mechanistic studies illustrate that, IL2-activated CD56$^+$ lymphocytes mediated immunomodulation occurs through direct cell-to-cell contact and TNFα, while the anti-viral effect is dependent on direct cell-to-cell contact.

Introduction

Dendritic cells (DCs) are professional antigen presenting cells and act as sentinels at the periphery. On viral infection, these cells undergo maturation and activate a cascade of both innate and adaptive anti-viral immune response. DCs can act as carriers of viral load (Trojan horse) and are susceptible to virus-mediated modulation (Rinaldo and Piazza, 2004). Apart from DCs, natural killer (NK) and natural killer T (NKT) cells are CD56$^+$ innate lymphocytes that bridge the gap between the innate and adaptive system. These cells contribute to DCs maturation through immunomodulation, and provide primary anti-viral defense (Biron and Brossay, 2001; Münz et al., 2005). NK-DC interactions are bi-directional and can occur at various sites including the site of inflammation (Cooper et al., 2004; Moretta, 2005). Therefore, interaction of DCs with innate lymphocytes represents a major control mechanism in limiting the virus infection at the periphery before adaptive response sets-in.

Japanese encephalitis virus (JEV) is a mosquito-borne, ssRNA (~11 kb, monopartite, linear) virus belonging to family *Flaviviridae* and genus *Flavivirus*. JEV remains as one of the major causative agents of pediatric encephalitis, and is mainly prevalent in South East Asian countries and the Pacific's (Erlanger et al., 2009). In humans, clinical presentation of JEV infection ranges from mild febrile illness to severe meningoencephalitis (Solomon, 2003). However, in JEV endemic region most of the JEV infections manifest in mild febrile, sub-clinical disease (http://www.cdc.gov/ncidod/dvbid/jencephalitis/facts.htm) and leading to protective adaptive response (Kumar et al., 2004).

During viral infection, both virus virulence factor and host factors appear to influence the pathogenesis. In vivo and in vitro studies have shown that JEV replicates in macrophages, and human mononuclear leukocytes (Aleyas et al., 2009; Mathur et al., 1988; Kedarnath et al., 1986). Recently, two groups have independently shown that wild-type JEV impairs the functional status of mouse DCs and thus leading to poor T cell response (Aleyas et al., 2009; Cao et al., 2011). In several *flavivirus* infections such as West Nile (WNV), Hepatitis C virus and dengue virus (DENV), NK and NKT cells are known to reduce virus load (Ye et al., 2009; Zhang et al., 2010) and curtail viral spread (Shresta et al., 2004). In humans, an increase in early NK and NKT activation is associated with mild dengue disease (Azeredo et al., 2006). However, the role of NK and NKT cells in modulating DCs maturation and anti-viral effect during JEV infection remains unresolved.

The aim of our study was to evaluate (a) the interaction between JEV, human monocyte derived DCs (MDDCs) and CD56$^+$ cells (NK+NKT) in vitro; and (b) differential ability of different JEV strains to induce and/or respond to this interaction. We used two JEV strains—JE057434 (neurovirulent, primary

* Corresponding author. Fax: +91 20 2587 1895.
E-mail address: gore.milind@gmail.com (M. Gore).

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clinical isolate, as wild-type strain) and SA14-14-2 (non-neurovirulent, live-attenuated, commercial, JEV vaccine strain) to understand the effect of viral virulence in modulating DC-$CD56^+$ cell interaction. Our results indicate the replication of JEV in immature-MDDCs and subsequent activation and maturation of these cells. We also confirm the contribution of $CD56^+$-NK and -NKT cells in increased DCs maturation and an overall decrease in JE virus load.

Results

Genetic and phenotypic characterization of JE057434 and SA14-14-2

JE057434 (wild-type) was isolated from human clinical sample obtained from Gorakhpur region of India in 2005. Comparison of both nucleotide and protein sequences of JE057434 (GenBank ID: EF623988.1) and SA14-14-2 (GenBank ID: AF315119.1) was performed using MEGA5 software. Both strains belonged to genotype III and exhibited a 97.48% genome similarity. A total of 276 nucleotides and 53 amino acid differences were observed between both the viruses. The major nucleotide and amino acid changes within the coding regions of both virus genomes are shown in Table 1. Prime changes, between JE057434 and SA14-14-2, at E138K, I176V, Q264H, K279M, A315V, and K439R in E protein, E63D in NS2B, A105G in NS3, and I106V in NS4B resembled the mutations observed between SA14 (GenBank ID: M55506.1) and SA14-14-2 E and NS regions. JE057434 was neurovirulent in mice.

Anti-flavivirus immune status

The donor's sera were screened for neutralizing antibody (N-Ab) against DENV, WNV, and JEV. Ninety percent of the donors were seropositive for either of the *flavivirus* tested, predominantly to WNV or DENV. The geo-mean $\pm$ SEM of N-Ab titer for DENV = 35.45 ± 26.93 (range: 7–896); WNV = 84.87 ± 11.76 (range: 28–336); and JEV = 17.02 ± 1.08 (range: 7–56). Moreover, in humans, anamnestic anti-*flavivirus* immune response is also known to contribute to protection from severe JE disease, rather than causing antibody dependent enhancement as seen in secondary dengue infections (Libraty et al., 2002; Solomon, 2004).

Throughout our study, in vitro JEV infection of immature-MDDCs (im-MDDCs) or mature-MDDCs (m-MDDCs) was carried out in complete absence of external neutralizing antibody or donor's autologous sera. Therefore, we assume that there is no possibility of antibody dependent enhancement influencing the growth kinetics or other response induced by the JEV strains during these experiments.

JEV replicates in im-MDDCs but not in m-MDDCs

DCs are known to support *flavivirus* replication. Both, wild-type and vaccine JEV strains replicated efficiently in im-MDDCs; while, m-MDDCs failed to support replication of JEV strains (Fig. 1A). At 48 h post-infection (hpi), both JEV strains reached a titer of 6 $\log_{10}$ in im-MDDCs, while it remained 1 $\log_{10}$ in m-MDDCs cultures. Growth kinetics experiment on im-MDDCs showed that both wild-type and vaccine JEV strains did not vary in their replication potential. Both JEV strain attained titers with peak at 48 hpi (approx 6 $\log_{10}$) and were maintained until 96 hpi, by 192 hpi the viral load (approx 3 $\log_{10}$) decreased to basal levels (Fig. 1B).

Flaviviruses use receptor-mediated endocytic pathway to gain entry into the host cell (Smit et al., 2011; Chu and Ng, 2004) and the endocytic ability of the DCs diminishes on their maturation (Guermonprez et al., 2002). Therefore, we speculated that the difference in im- and m-MDDCs to support JEV might be based on variation in phagocytic potential or other markers. As a preliminary experiment, blockage of endocytic ability of im-MDDCs using non-specific inhibitors such as Phenylarsine oxide (PAO) or Nocodazole and Cytochalasin B resulted in reduced FITC-Dextran uptake and rendered im-MDDCs less susceptible to JEV growth (Fig. S1).

Table 1
Nucleotide and amino-acid differences between wild-type (JE057434) and vaccine (SA14-14-2) JEV strain genome coding region.

Position	Nucleotide	Amino acid
C[a]	T292C	L66S
	A441G	T116A
prM	G648A,C649T	A58M
	C841T	T122M
E	C1296T	L107F
	G1389A	E138K
	A1458G	T161A
	A1503G	I176V
	A1506G	T177A
	A1708G	E244G
	G1769T	Q264H
	A1813T	K279M
	C1921T	A315V
	G2064A	A363T
	G2170A	R398K
	A2293G	K439R
	G2317A	G447D
NS1	T3165A	S230T
	G3181A	G235D
	T3216A	L247I
	G3351A	G292S
	C3489G,C3491T	L338V
	G3493T	R339M
	G3528C	D351H
	T3539A	N354K
	C3652T	A392V
NS2A	G3972A, T3974C	A84T
	A4085C	Q121H
NS2B	G4403T	E63D
	A4408G	D65G
	G4475C	L87F
NS3	A4782G	M59V
	C4921G,T4922C	A105G
	A5114C	E169D
	C5172A	L189M
	C5311T	A235V
	G5656A	G350E
NS4A	NIL	NL
NS4B	A7227G	I106V
	C7243T	S111F
	T7263C	V118A
NS5	T7683C	S3P
	C7768G	A31G
	C7809A	R45S
	G7821A	V49I
	G8261T	M195I
	A8490G	I272V
	C8832T	H386Y
	G9593T	Q639H
	T9688C	V671A
	G9954C	A760P
	C9978G	L768V
	A10081T	K802I
	G10371A	V899I

[a] C—Capsid; prM—pre-membrane; E—envelope; NS—non-structural.

Productive JEV infection brings about activation of im-MDDCs

On pathogen exposure DCs are activated, and efficiently modulate adaptive response through expression of surface

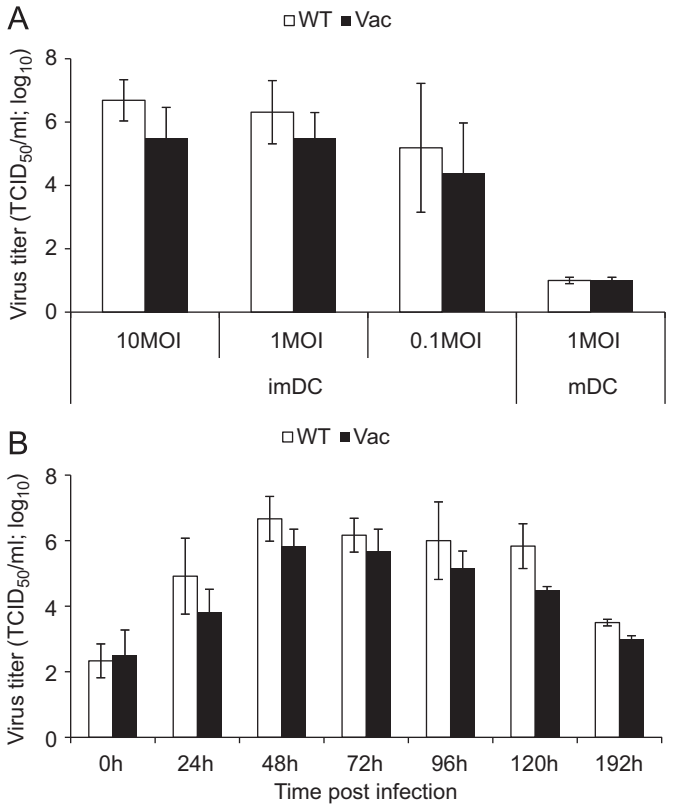

Fig. 1. im-MDDCs, but not m-MDDCs, support productive JEV replication. (A) Infectious virus levels, at 48 hpi, in cell-culture supernatant of im-MDDCs and m-MDDCs infected with wild-type (WT; open bars), or vaccine (Vac; filled bars) JEV. (B) Growth kinetics of WT and Vac JEV in im-MDDCs culture. Data represents mean ± SD obtained from three independent experiments.

co-stimulatory molecules and cytokines/ chemokines. At 48 hpi, in im-MDDCs, both JEV strains were able to up-regulate several co-stimulatory markers (CD −83, −86, −80, and 40, HLA-A/B/C, HLA-DR, and ICAM-1) (Fig. 2A). Wild-type JEV strain infection of im-MDDCs also led to modulation in phagocytic index of the cells (Fig. 2B). However, JEV infection failed to up-regulate CD86 levels on m-MDDCs (Fig. S2). We had observed from our earlier experiments, with human macrophages, that most of the immunomodulatory effects of JEV were dependent on virus replication (unpublished data). Thus, we used beta-propiolactone inactivated JEV to determine whether the JEV replication is needed to up-regulate co-stimulatory markers on im-MDDCs. The inactivated JEV failed to induce CD86, CD83 and HLA-DR on im-MDDCs (Fig. 2C). Taken together, these data indicate both wild-type and vaccine JEV strains were similar in their ability to induce im-MDDCs maturation and it is dependent on virus replication.

Apart from the co-stimulatory markers, at 48 hpi, JEV infection also resulted in up-regulation of several cytokines and chemokines levels, as compared with un-infected im-MDDCs culture supernatant (Fig. 3). The prominent cytokines and chemokines induced by both JEV strains were type-I IFN, interleukin (IL)-6, CCL2, CXCL10, and CCL5 (Fig. 3). However, they varied in their ability to induce tumor necrosis factor (TNF)-α and IL8. Wild-type JEV strain induced a significantly higher TNFα ($p=0.017$) and IL8 ($p=0.027$) levels as compared with un-infected cultures, while vaccine JEV failed to do so. JEV infection also resulted in insignificant IL10 release (Fig. 3) and IL12p70 levels remained undetectable (data not shown); while, inactivated JEV failed to induce type-I IFN from im-MDDCs (data not shown).

PI3K and p38 pathways influences DC activation during JEV infection

We further characterized the pathway that was involved in DCs maturation during JEV infection. In accordance with earlier studies (Aleyas et al., 2009; Chang et al., 2006), blocking of phosphatidylinositol-3-kinase (PI3K) and p38 using specific inhibitors (LY294002 and SB203580, respectively) resulted in reduced type-I IFN, TFNα, IL8 (Fig. 4A), and CD86 (Fig. 4B) levels during JEV infection. While, IL6 level was dependent on PI3K pathway rather than p38 pathway (Fig. 4A) during JEV infection of im-MDDCs. Interestingly the virus levels in all the tested conditions remained unaltered (Fig. 4C).

IL2-activated CD56$^+$ cells impart immunomodulation via cell-to-cell contact and TNFα

During in vivo condition, apart from virus-DC interaction, CD56$^+$ lymphocytes (NK/ NKT) also determine the course of immune response. Among the different co-stimulatory molecules determined CD86 showed highest MFI (mean fluorescence intensity) increase post-JEV-infection (Fig. 2A) thus in all further experiments levels of CD86 were used as MDDCs maturation maker.

Presence of IL2-activated CD56$^+$ cells, resulted in DC maturation as evident from increased CD86 (Fig. 5A), TNFα, IL6, IL1β, and CXCL9 levels (Fig. 5B). However, in the same experiments type-I IFN levels decreased (Fig. 5B). Conversely, resting CD56$^+$ cells were unable to modulate CD86 levels and most of the cytokine or chemokine tested, with an exception of IL6. Co-culturing of resting CD56$^+$ cells led to an increased IL6 levels in un-infected and vaccine-JEV infected im-MDDCs cultures ($p=0.027$) (Fig. 5B). Both IL12p70 and IFNγ levels remained undetectable in JEV-infected im-MDDCs cultures, even during the co-culture experiments (data not shown).

We then determined whether CD56$^+$-cells-mediated DC maturation was dependent on cell-to-cell contact—or cytokines (TNFα). Our results showed IL2-activated CD56$^+$ cells induced im-MDDCs maturation partially through direct cell-to-cell contact and TNFα, as separation of the cells by a permeable membrane (transwell) and presence of neutralizing antibody against TNFα abrogated DC maturation (Fig. 6A and B).

CD56$^+$ cells mediated anti-viral effect dependent on cell-to-cell contact and E:T ratio

IL2 treatment is known to augment NK cell killing ability (Grimm et al., 1982) and cytolytic ability of NK is known to correlate with its degranulation (i.e., CD107a) levels (Alter et al., 2004). As expected, the IL2-activated CD56$^+$ cells showed higher degranulation capacity resulting in increased DC killing (Fig. 7A and B) and greater ability to reduce JE viral load (Fig. 5C) than resting CD56$^+$ cells. The IL2-activated CD56$^+$ cells mediated JE virus level reduction through direct cell-to-cell contact (wild-type JEV = 5.1 ± 0.25 $\log_{10}$, vaccine JEV = 3.5 ± 0.57 $\log_{10}$), as separation of the virus-infected cells and IL2-activated CD56$^+$ cells by transwell did not result in viral load reduction (Fig. 8A). In addition, it was independent of TNFα, as presence of neutralizing antibody against TNFα during co-culture experiments did not result in increase in JEV titers (wild-type JEV = 5 ± 0.1 $\log_{10}$, vaccine JEV = 3.8 ± 0.28 $\log_{10}$).

The decrease in viral load was more evident in vaccine-JEV infected im-MDDCs cultures as compared with wild-type JEV strain. There was approximately 2 $\log_{10}$ reduction in vaccine JE virus level while only ~1 $\log_{10}$ reduction in wild-type JE virus level by IL2-activated CD56$^+$ cells. The virus level reduction was also dependent on CD56$^+$ cell number in culture. At higher E:T ratio (5:1), in IL2-activated CD56$^+$ cells/DC co-cultures, there was increased DC lysis (Fig. 7B), and wild-type virus level reduction was more predominant. While, resting CD56$^+$ cells were able to exert a minimal anti-viral effect even at higher E:T ratio (5:1) (Fig. 8B).

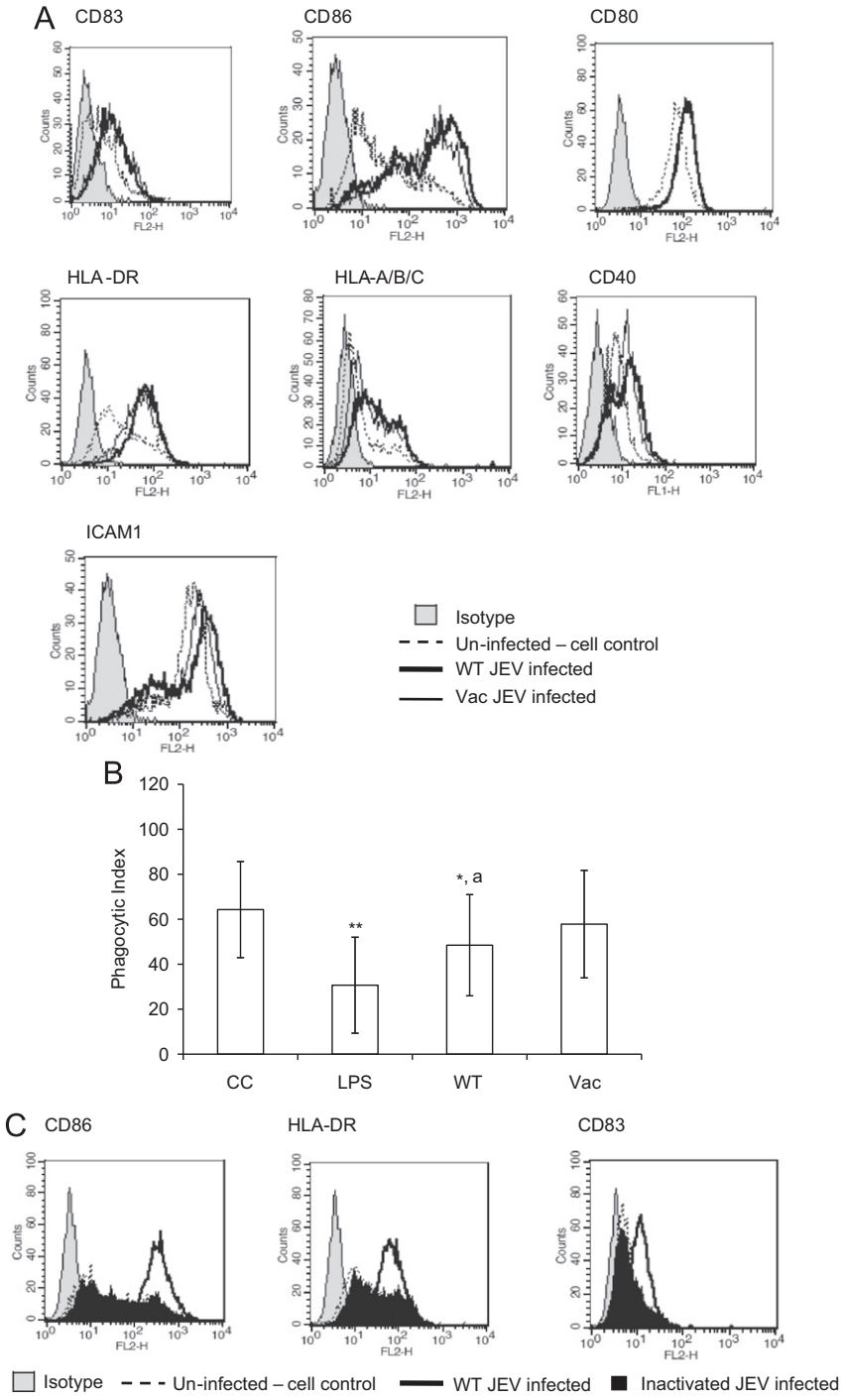

Fig. 2. Replicating JEV induces DCs maturation. (A) and (C) Histogram overlay representing various co-stimulatory molecule levels on un-infected control (broken line), wild-type JEV-infected (thick line), vaccine JEV-infected (thin line), and (C) inactivated-JEV infected (black-filled line) im-MDDCs cultures. Data shown are representative of three independent experiments. Gray-filled line represents isotype-matched control. (B) Phagocytic index levels in JEV infected im-MDDCs. CC—un-infected im-MDDCs, LPS—LPS-treated im-MDDCs (positive control), WT—wild-type JEV-infected im-MDDCs, and Vac—vaccine JEV-infected im-MDDCs. Data represents mean $\pm$ SD of four independent experiments. Statistical analysis was done using paired student *t*-test. *, $p \leq 0.05$ and **, $p \leq 0.01$ as compared with un-infected (CC) im-MDDCs; [a], $p \leq 0.05$ on comparison between wild-type (WT) and vaccine (Vac) JEV-infected im-MDDCs cultures.

Discussion

Interplay between the pathogen, DCs and innate lymphocytes plays a crucial role during initial stages of infection. In the current study, we have tried to understand the interaction between human MDDCs, and NK+NKT ($CD56^+$ cells) during wild-type/vaccine JEV infection in vitro. Our results showed productive JEV replication results in im-MDDCs activation via PI3K and p38 pathways, and presence of IL2-activated $CD56^+$ cells imparts both immunomodulatory and anti-viral effect.

Cells of myeloid lineage are known to support JEV (Aleyas et al., 2009), and viral replication potential forms a major virulence factor (Hase et al., 1993). We observed that human im-MDDCs supported a productive JEV replication and a

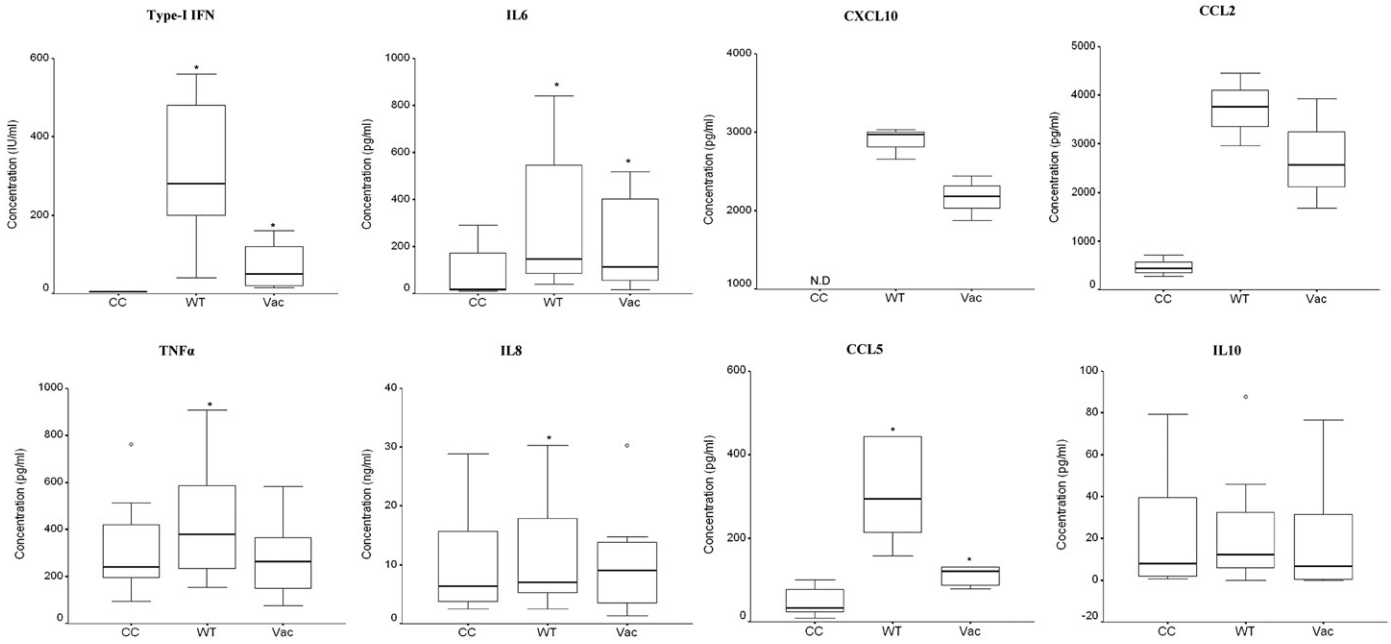

Fig. 3. Cytokine and chemokine levels. Cytokine and chemokine levels in un-infected (CC), wild-type JEV (WT), or vaccine JEV (Vac) infected im-MDDCs culture supernatant ($n=7$ donors). Box-plots represent minimum, first quartile, median, third quartile, and maximum. "o" in box-plot represents extremes and outliers. *, $p \leq 0.05$; Wilcoxon rank test, compared with CC. N.D—negligible detectable limit (< 10 pg/ml).

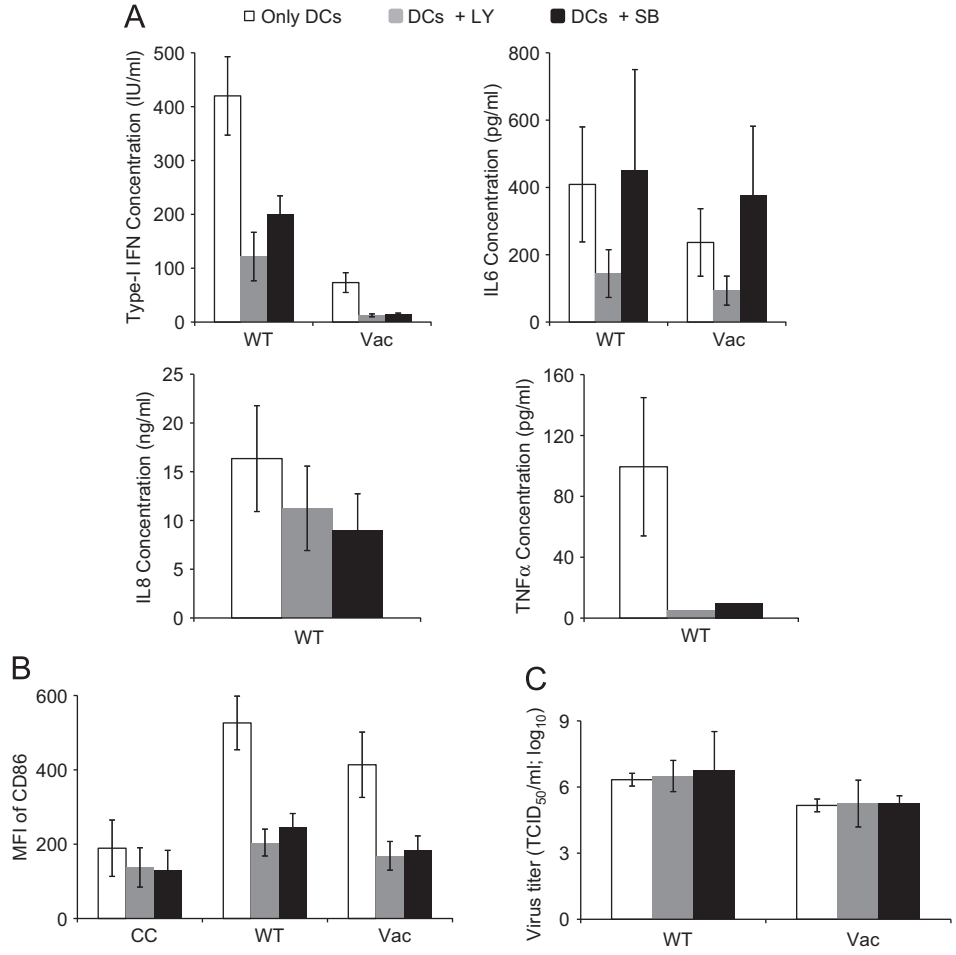

Fig. 4. PI3K and p38 pathway influence DC activation, but not virus replication during JEV infection. Wild-type JEV (WT), or vaccine JEV (Vac) infected or un-infected (CC) im-MDDCs controls were cultured in the presence or absence of 5 μM PI3K inhibitor—LY294002 (LY), or p38 inhibitor—SB203580 (SB). (A) Cytokine levels in cell culture supernatant (mean ± SEM), (B) CD86 levels on im-MDDCs and (C) virus titers ($TCID_{50}$/ml) in cell-culture supernatant. Data in panel (B) and (C) represents mean ± SD of three independent experiments.

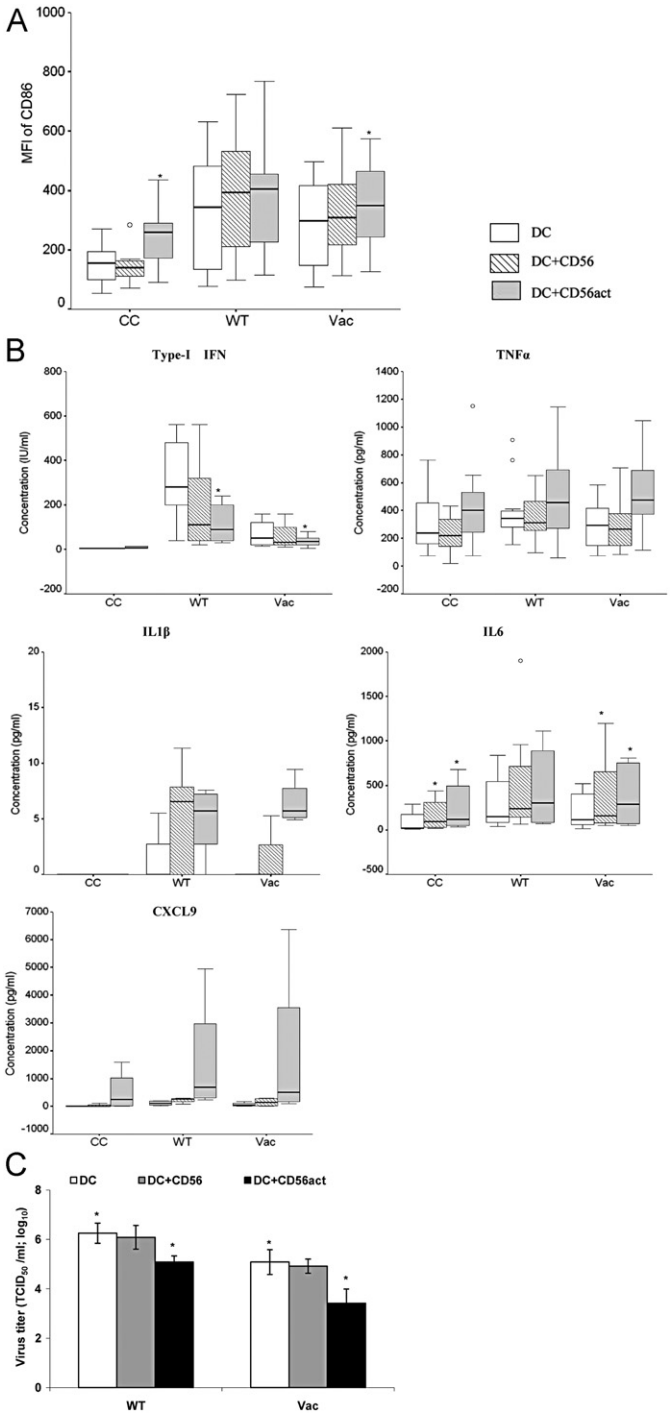

Fig. 5. Presence of IL2-activated CD56$^+$ cells exerts immunomodulation and anti-viral effect. Un-infected (CC), wild-type (WT), or vaccine (Vac) infected im-MDDCs were cultured in medium alone or co-cultured with resting CD56$^+$ (DC+CD56) cells or IL2-activated CD56$^+$ (DC+CD56act) cells at 1:1 ratio for 48 h. (A) CD86 levels on im-MDDCs ($n=7$ donors), (B) cytokine and chemokine profile ($n=7$ donors, CXCL9=4 donors), and (C) virus titer ($TCID_{50}$/ml) on culture supernatant ($n=6$ donors). Data is represented as, (A) and (B) box-plots or (C) mean ± SD. Box-plots represent minimum, first quartile, median, third quartile, and maximum. "o" in box-plot represents extremes and outliers. For (A) and (B) *, $p \leq 0.05$; Wilcoxon rank test; for (C) *, $p \leq 0.05$; paired Student *t*-test, as compared with DC.

subsequent DC activation; while, m-MDDCs failed to support replication of JEV strains. The phenomenon of refractive viral replication in m-MDDCs has been reported during vaccinia (Engelmayer et al., 1999), DENV (Wu et al., 2000) and HIV infections. The mechanism is more thoroughly studied in HIV cis-infection, and it has been attributed to inhibition of viral entry/fusion (Cavrois et al., 2006) or viral replication (Granelli-Piperno et al., 1998; Bakri et al., 2001). The exact mechanism behind refractive *flavivirus* replication in m-MDDCs is not yet clearly understood. During *flavivirus* infection, receptor-mediated endocytic pathway forms crucial mechanism for viral entry into the host cell (Smit et al., 2011; Chu and Ng, 2004). Our preliminary results also indicated that blockage of endocytic ability of im-MDDCs renders them less susceptible to JEV growth (Fig. S1). Therefore, JEV entry and replication in MDDCs might depend on DC maturation state, with endocytic ability of the DC playing an important role, rather than viral virulence.

Flaviviruses are known to induce DCs maturation (Dejnirattisai et al., 2008; Ho et al., 2001; Li et al., 2011; Silva et al., 2007) and PI3K and p38 pathways regulate virus specific immune response (Mogensen and Paludan, 2001). As expected, JEV infection resulted in im-MDDCs maturation and it was dependent on virus replication, PI3K and p38 pathway. However, in contrast to in vitro study on murine DCs (Aleyas et al., 2009; Cao et al., 2011) our data suggested that the Indian wild-type (JE057434) JEV strain did not inhibit DCs maturation, rather it had similar ability as that of vaccine JEV to induce co-stimulatory markers in human im-MDDCs. This variation might be due to the difference in wild-type strain and/or the study model used in these studies. During JEV infection, PI3K pathway is known to regulate early apoptosis mechanism of the cell without hampering JEV replication (Lee et al., 2005). Likewise, our results also indicated that PI3K and p38 regulate immune modulation of DCs without affecting the JEV replication in im-MDDCs.

We also observed that, the wild-type JEV infection of im-MDDCs resulted in an elevated amount of cytokines and chemokines and induced a significantly higher pro-inflammatory cytokines (TNFα and IL8) than vaccine JEV. Some of the JEV-induced cytokines/chemokines, such as CXCL10, CCL2, CCL5, and type-I IFN, impart immunoprotection (Glass et al., 2005, 2006; Klein et al., 2005), while TNFα and IL8 are known to be immunopathogenic (Ravi et al., 1997; Winter et al., 2004) to the host. Therefore, the ability of wild-type JEV strain to induce DC activation, along with elevated pro-inflammatory cytokine might potentiate a probable immunopathogenic reaction.

In vivo CD56$^+$ lymphocytes (NK/NKT) regulate DC maturation as well as provide primary anti-viral defense at the periphery (Biron and Brossay, 2001; Münz et al., 2005; Walzer et al., 2005). Our results also showed that JEV infection resulted in CCL5, and CCL2 release from im-MDDCs; these chemokine are known to facilitate NK-cell chemotaxis (Loetscher et al., 1996; Maghazachi et al., 1994) along with T cells and monocytes.

In accordance with previous reports (Gerosa et al., 2002, Piccioli et al., 2002) our results indicated that presence of IL2-activated CD56$^+$ cells, but not resting CD56$^+$ cells, resulted in DC maturation and it was dependent on cell-to-cell contact and TNFα. It has also been shown that PMA-activated T cells aid in DC maturations during DENV infection (Dejnirattisai et al., 2008). Therefore, it is possible that apart from innate lymphocytes other cells can also contribute to DC maturation during flaviviral infection.

Gerosa et al. (2005) have shown that co-culturing of IL2-activated NK with stimulated DCs result in increased IFNα. However, in our study, type-I IFN levels decreased, while TNFα levels increased during co-culturing of JEV-infected DCs with IL2-activated CD56$^+$ cells. Type-I IFN and TNFα are known to cross-regulate each other during certain disease conditions (Palucka et al., 2005). Hence, we speculated that the decreased type-I IFN might be due to increased TNFα levels. Nevertheless, neutralizing TNFα, during JEV-infected im-MDDCs—CD56$^+$ cells

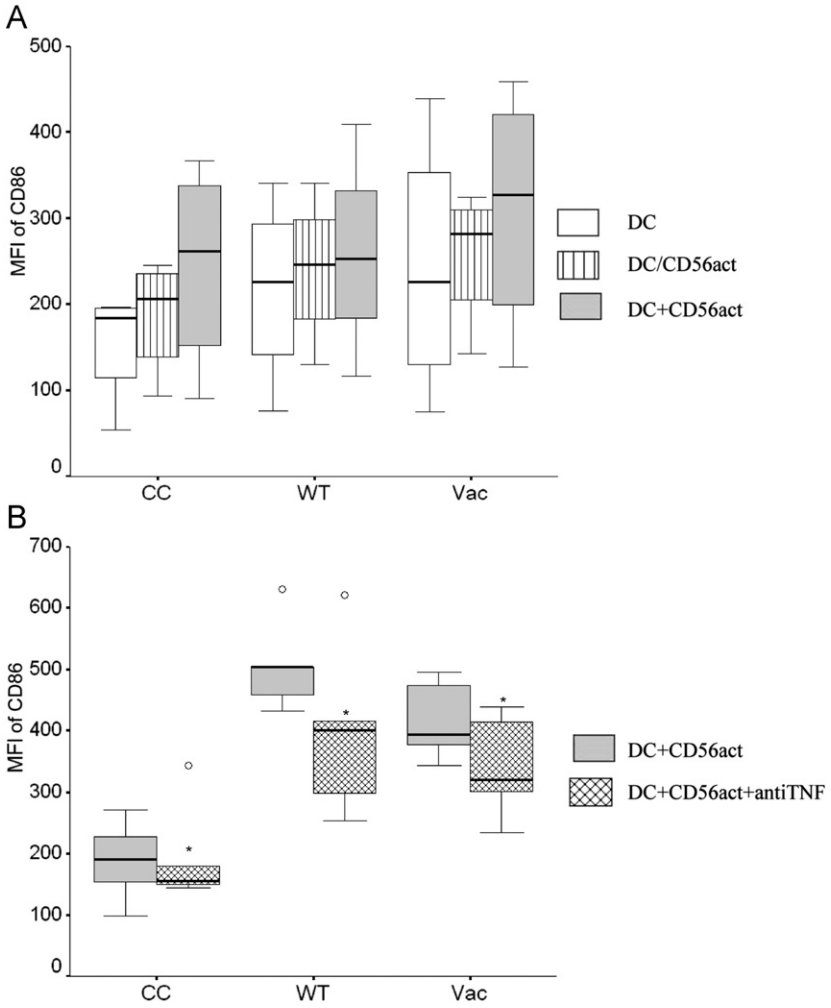

Fig. 6. Immunomodulatory effect of IL2-activated CD56$^+$ cells occurs through cell-to-cell contact and TNFα. Box-plot representing CD86 levels in un-infected (CC), wild-type (WT), or vaccine (Vac) infected im-MDDCs, when co-cultured (A) in direct contact with IL2-activated CD56$^+$ (DC+CD56act), or in transwell setup (DC/CD56act) ($n=4$ donors); (B) direct co-culturing in the presence (DC+CD56act+antiTNF) or absence (DC+CD56act) of anti-TNFα ($n=5$ donors). Box-plots represent minimum, first quartile, median, third quartile, and maximum. "*o*" in box-plot represents extremes and outliers. *, $p \leq 0.05$; Wilcoxon rank test, as compared with corresponding DC+CD56act.

co-cultures, did not restore type-I IFN levels (data not shown). Therefore, the decrease in type-I IFN levels could probably be due to direct lysis of IFN producing DCs by activated CD56$^+$ cells.

One of the major hallmarks during DC–NK interaction is production of IFNγ by NK cells. Its induction is dependent on IL12p70, IL15, IL18, E:T ratio and other factors (Ferlazzo et al., 2004; Walzer et al., 2005). However, during our co-culture experiments both IL12p70 and IFNγ levels remained undetectable. High level IL12p70 induction from human DCs requires two signals—CD40-40L interaction and IFNγ (Snijders et al., 1998). Role of external IFNγ to induce IL12p70 from im-MDDCs has been demonstrated during DENV infection (Libraty et al., 2001). Other studies have also shown high levels of type-I IFN can inhibit IL12p70, and consequently modulate IL12/IFNγ axis (McRae et al., 1998; Byrnes et al., 2007). Therefore, more studies would be essential to understand the inability of JEV to induce IL12p70/IFNγ from DC or NK/NKT cells as it involves multiple regulatory parameters.

Overall, the ability of CD56$^+$ cells to reduce JE-viral load was independent of TNFα, and primarily dependent on (i) CD56$^+$ cell activation status, (ii) direct cell-to-cell contact and (iii) E:T ratio. On IL2-activation, there was an increased basal level degranulation of CD56$^+$ cells and more effective target-cell lysis. It is known that at higher E:T (NK/DC) ratio there is potent killing of autologous DCs (Piccioli et al., 2002), and mature NK-cells mediate direct-cytolysis through $Ca2^+$ dependent granule exocytosis pathway or Fas ligand (Zamai et al., 1998). Therefore, CD56$^+$ cell mediated contact-dependent target lysis might play an important role in controlling JE-virus load/spread in host tissues.

Interestingly, both JEV strains varied in their susceptibility towards CD56$^+$ mediated anti-viral effect, even though activated-CD56$^+$ mediated cell death remained similar in both JEV infected im-MDDCs cultures. Therefore, this variation in anti-viral susceptibility might be due to the intrinsic property of the virus. Such variation in viral strain sensitivity to innate anti-viral state is observed with other *flavivirus* (Aguilar et al., 2005). Moreover, in wild-type JEV infected cultures, increase in DC-CD86 levels during IL2-activated CD56$^+$ cells co-culture varied from donor to donor, and was not as prominent as observed in un-infected and vaccine JEV infected cultures. Therefore, it is alluring to speculate that wild-type virus might undermine DC—IL2-activated CD56$^+$ cell interactions in few individuals.

Even though our in vitro results might not fully reflect the in vivo scenario, it offers a preliminary insight into the interaction of JEV with human DCs and NK/NKT cells. Based on our over-all findings, we presume that the decreased cytokine levels and

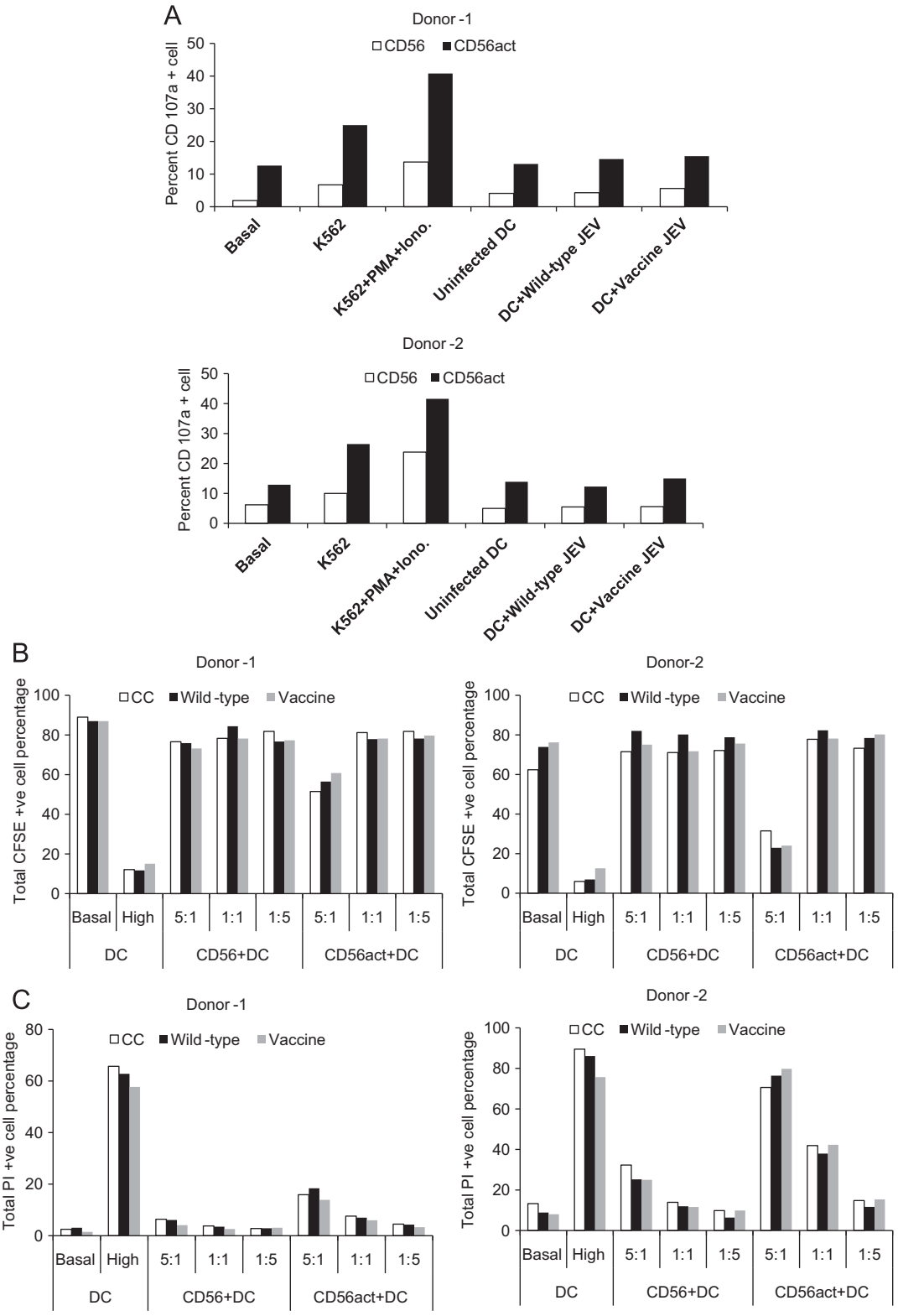

Fig. 7. IL2-activated CD56$^+$ cells exhibited increased degranulation and DC killing ability. Two donor data-set of (A) CD107a levels in resting CD56$^+$ (CD56, open bars) and IL2-activated CD56$^+$ (CD56act, filled bars) cells cultured in medium alone, or co-cultured with K562, K562 + PMA + ionomycin (positive control), un-infected/JEV-infected im-MDDCs at 1:1 ratio. CD56$^+$ cell mediated DC lysis was measured by CFSE-based cytotoxicity assay on CFSE-labeled DCs. (B) Total viable target-cell levels—CFSE$^+$ DC percentage, and (C) total dead target-cell levels—PI$^+$ DC percentage. In panel (B) and (C) high-positive control—triton-X100 (0.1%) treated target-cells.

increased susceptibility towards innate anti-viral effects of attenuated vaccine JEV might facilitate efficient virus clearance and thus preventing virus spread into other tissues.

In conclusion, interaction between JEV, im-MDDCs and IL2-activated CD56$^+$ cells is complex and would depend on both viral and host factors. During JEV infection, DC/CD56$^+$ cell

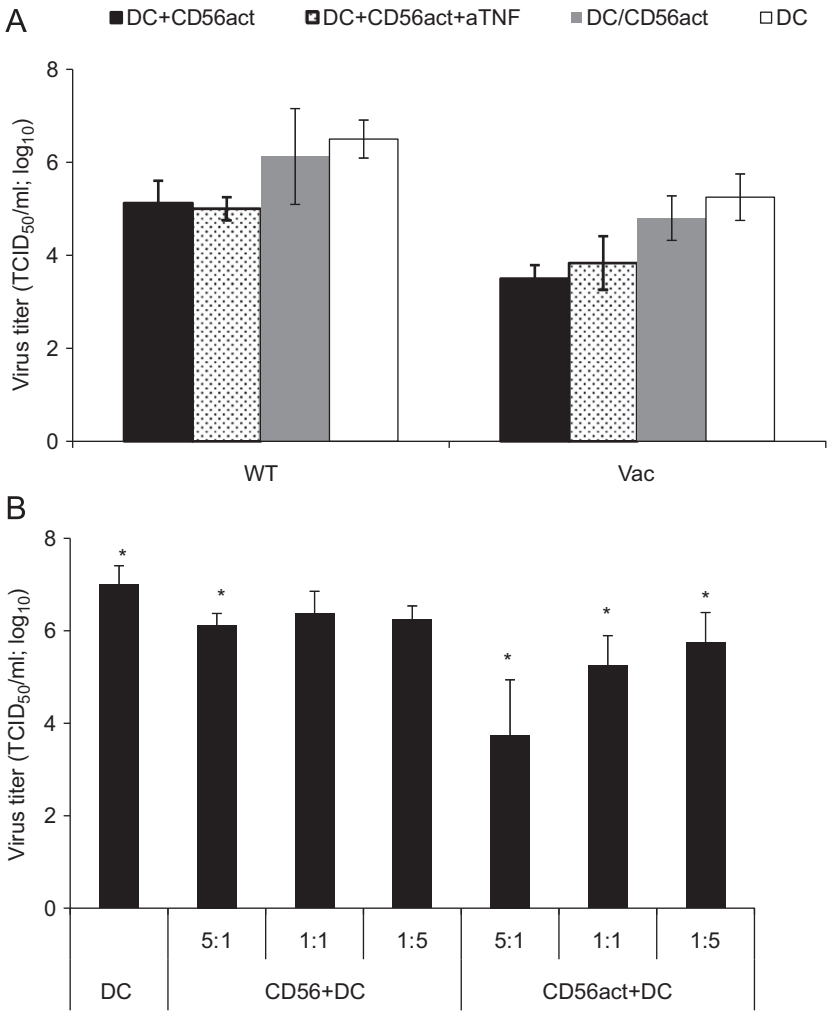

Fig. 8. IL2-activated $CD56^+$ mediated anti-viral effect dependent on cell-to-cell contact and E:T ratio. (A) $TCID_{50}$ levels in wild-type JEV (WT), or vaccine-JEV (Vac) infected im-MDDCs cultured separately (DC) or co-cultured with IL2-activated $CD56^+$ (DC+CD56act), and in presence of antiTNFα (DC+CD56act+antiTNF), or in transwell setup (DC/CD56act) ($n=4$ donors). (B) Reduction in virus load at various CD56: DC (E:T) ratio ($n=2$ donors). Data represents mean ± SD, * $p \leq 0.05$; paired Student *t*-test, as compared with DC.

interaction might form one of the key factors in curtailing JEV at the periphery while providing immunomodulation.

Materials and methods

Cells lines and virus

Cell lines derived from, baby hamster kidney (BHK-21) and human amnion (WISH) were maintained in Minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS, GIBCO, USA), 2 mM Glutamine, 100 U/ml Penicillin, and 100 μg/ml Streptomycin. Wild-type (JE057434; WT), vaccine (SA14-14-2; Vac) JEV strains and Encephalomyocarditis (EMC) virus were propagated in BHK21 and WISH cells, respectively, to generate working stocks. Aliquots of clarified virus stocks were stored at −80 °C. Stock virus was titrated by plaque assay.

Antibodies and reagents

Human IFNα (reference standard) was obtained from the National Institute for Biological Standards and Control (NIBSC, London). PI3K, and p38 inhibitor (LY294002 and SB203580, respectively) were procured from Sigma Aldrich, and stocks were prepared and stored according to the manufacturer's instructions. PE-conjugated anti-human CD80, CD86, HLA-DR, HLA-A/B/C, CD83, ICAM1 and CD14, FITC-conjugated CD40, CD107a and CD1a (BD Biosciences, USA) were used for flow-cytometric staining of cell surface markers. Anti-human–TNFα neutralizing antibody and recombinant human cytokines, Granulocyte-Monocyte colony stimulating factor (GM-CSF), IL4, and IL2 were procured from R&D Systems, USA.

Generation of immature and mature MDDCs

Buffy-coat packs from anonymous donations were obtained from Sahyadri Hospital Blood Bank (Pune, India), as approved by Institute (NIV, Pune) ethical committee. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll–Hypaque (Sigma, USA) gradient separation. $CD14^+$ cells were isolated from PBMCs by positive selection using CD14 MicroBead mAbs (Miltenyi Biotec, USA). To promote differentiation into im-MDDCs, 1×10^6 purified $CD14^+$ cells were cultured for 5–6 days in 5 ml of RPMI-1640 (GIBCO, USA) supplemented with 10% FCS, 2 mM Glutamine, 100 U/ml Penicillin, 100 μg/ml Streptomycin and in the presence of GM-CSF and IL4 (50 ng each). On day 3, half of the medium was replenished with fresh medium and cytokines. The purified im-MDDCs were stained with anti-CD14, CD80 and CD1a antibody on day 5; ~90% cells were $CD14^-CD1a^+CD80^+$. m-MDDCs

were generated by incubating the purified im-MDDCs with LPS (1 μg/ml) for 24 h.

Virus infection of MDDCs

1×10^6 im-MDDCs or m-MDDCs were infected with either wild-type or vaccine strain of JEV at a multiplicity of infection (MOI) of one (unless otherwise mentioned) in complete medium (RPMI-1640 +10% FCS) for 1 h at 37 °C. All virus infections were carried out in the absence of external neutralizing antibody or autologous plasma. The cells were then washed thoroughly with PBS to remove unadsorbed virus, and cultured in fresh complete media supplemented with GM-CSF and IL4. A parallel un-infected im-MDDCs control was also maintained. At 48 hpi, DC maturation levels and cytokine and chemokine levels in culture supernatants were estimated. Virus levels in culture supernatant were estimated by tissue culture infective $dose_{50}$ ($TCID_{50}$) assay. Titers were calculated as $TCID_{50}$/ml using Spearman–Kärber method.

DC–$CD56^+$ cells co-culture

Peripheral $CD56^+$ cells comprise both NK ($CD56^+CD3^-$) and NKT ($CD56^+CD3^+$) population of innate lymphocyte. $CD56^+$ cells were isolated from PBMCs by positive selection using CD56-Microbead mAbs (Miltenyi Biotec, USA). Isolated $CD56^+$ cells were used directly as resting cells or activated with IL2 (25 ng/ml) for 5 days to obtain IL2-activated $CD56^+$. DC-$CD56^+$ cross-talk were studied by co-culturing un-infected/JEV-infected im-MDDCs with autologous resting or IL2-activated $CD56^+$ cells at 1:1 ratio (unless mentioned). Briefly, the im-MDDCs were infected with JEV as mentioned above and washed thoroughly to remove unadsorbed viruses. One-hour post virus infection, the infected DCs were co-cultured with the $CD56^+$ cells. During the transwell (0.4 μ pore-size) experiments, the un-infected/JEV-infected im-MDDCs were maintained in lower chamber while IL2-activated $CD56^+$ cells were seeded in the upper chamber. DC maturation levels and cytokine and chemokine levels in culture supernatants were estimated at 48 hpi. Degranulation assay (CD107a levels) to estimate $CD56^+$ cells cytotoxic activity was performed according to protocol described by Alter et al., 2004. Briefly, the cells were co-cultured as mentioned above along with anti-CD107a-FITC (10 μl/ml), monensin (6 μg/ml), and brefeldin (1 μg/ml), CD107a levels in $CD56^+$ cells were estimated at 6 hpi. K562 and K562+PMA+ionomycin stimulus were used as known positive controls.

Flow-cytometry analysis

After 48 h post JEV infection, im-MDDCs were harvested, washed and resuspended in PBS with 0.5% BSA and 0.01% NaN_3, followed by incubation with PE-conjugated mAbs for 30 min at 4 °C. Cells were then washed and fixed in 1% paraformaldehyde in PBS. In each experiment, isotypic controls were used. Phagocytic index was calculated based on percentage cells positive for FITC-Dextran (FD40S, Sigma-USA) uptake. Cells were acquired using the FACS-Calibur and data analyzed using CellQuest Pro software (BD Biosciences, USA).

CFSE-based cytotoxicity assay

After washing with PBS the un-infected and JEV-infected im-MDDCs (one-hour post virus adsorption) suspensions were resuspended at 2×10^6 cells/100 μl and labeled with 2 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen, USA) for 30 min at 37 °C. The reaction was stopped by the addition of ice-cold PBS, followed by 5–10 min incubation. After two washes the CFSE-labeled target were resuspended in assay medium and cell concentration was adjusted to 2×10^5 cells/ml, and was plated in 24-well microtiter plates. The cells were then cultured at 37 °C and 5% CO_2 in the presence or absence of IL2-activated- or resting-$CD56^+$ cells, at various effector ratios. After 48 hpi, the cells were harvested and to stain for dead cells, propidium iodide (0.5 μg/tube) was added, and samples were mixed properly and directly analyzed by flow cytometry. Triton-X100 (0.1%, final concentration) treated cells were used as high-positive control.

Determination of type-I IFN and pro-inflammatory cytokine and chemokine levels

Bioassay was employed to determine active type-I IFN levels. Briefly, two fold dilutions of JEV infected im-MDDCs culture supernatants or hIFNα standards (1000 IU/ml, NIBSC) were added onto pre-formed monolayer of WISH cells. After 18 h of incubation, the cells were infected with EMC virus. Type-I IFN titers were expressed as IU/ml and were defined as the reciprocal value of the dilution of sample that showed a 50% reduction in virus induced cytopathic effect. The lowest detectable type-I IFN concentration was between 3–10 IU/ml. Cytokine (IL1β, IL6, IL8, IL10 and IL12p70) and chemokine (CCL2, CCL5, CXCL10, and CXCL9) levels on JEV-infected and un-infected im-MDDCs culture supernatants was determined using CBA kit (BD Biosciences). TNFα levels were estimated using DuoSet ELISA (R&D Systems).

Determination of anti-flavivirus antibody

Donors' sera were screened for the presence of N-Ab against DENV, WNV, and JEV. In vitro neutralization test was performed by incubating three-fold serially diluted donor's sera samples with 100 $TCID_{50}$ of the test virus on BHK-21 cell-line. N-Ab titer of serum samples was expressed as the reciprocal of the dilution at which 50% of virus added was neutralized; a titer of 1:10 was considered evidence of prior exposure.

Statistical analysis

Data is represented as bar-graphs representing mean ± SD, or boxplot. Statistical analysis was performed by paired-Student's *t*-test, or Wilcoxon signed-rank test for paired comparison, using SPSS software (version 11). Differences at $p \leq 0.05$ were considered statistically significant.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2012.05.013.

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